

polypeptide/secretin family (H.-C.Fehmann, R. Goke, B. Goke, Endocrine Reviews, 16, 390 (1995)) are indicated by asterisks; the hcrt1 and hcrt2 consensus residues (RL LL GNHAAGILT G; SEQ ID NO: 20) appear above the alignment. --

Rewrite the paragraph beginning on page 4, line 30, to read:

-- Fig. 6 shows the cDNA (SEQ ID NO: 5) and amino acid sequence (SEQ ID NO: 29) of clone 29. --

Rewrite the paragraph beginning on page 12, line 25, to read:

-- The complete coding nucleotide sequence, clone 35, of rat H35 cDNA is 569 nucleotides in length, and is listed in SEQ ID NO: 3. The complete preprohypocretin cDNA clone presents a 390 nucleotide open reading frame (ORF) plus triplet termination codons (second line in FIG. 5 and SEQ ID NO: 14). There is a N-terminal signal peptide with a cleavage site after nucleotide position 172 of SEQ ID NO:3. --

Rewrite the paragraph beginning on page 47, line 24, to read: _

-- In situ hybridization on coronal sections of brain from adult male rats was performed using the inserts from clones representing all four classes (A-D): 6, 10, 20, 21, 29 and 35. For all clones, the hybridization pattern was consistent with the Northern blot data. In the A class, the clone 35 mRNA displayed a striking pattern of bilaterally symmetric expression restricted to a few cells in the paraventricular hypothalamic area and ependymal cells surrounding the brain ventricles. No clone 35 signals were detected outside the hypothalamus. The sequence of clone 35 is shown in Fig. 5 and SEQ ID NO: 14. - -

Rewrite the paragraph beginning on page 50, line 19, to read:

-- DNA sequence analysis of the complete 569 nucleotide rat clone 35 revealed that the clone mRNA encodes a 130-residue putative secretory protein (called H35) or hypocretin with 4 sites for potential proteolytic maturation (FIG. 5 and see SEQ ID NO: 14). Several proteolytic fragments have been identified, some replacing C-terminal glycines with

amide groups. Two of the products of proteolysis have 14 amino acid identities across 20 residues. This region of H35 includes a 7/7 match with a region of the gut hormone secretin, suggesting that the prepropeptide gives rise to two peptide products that are structurally related both to each other and to secretin. --

Rewrite the paragraph beginning on page 50, line 28, to read:

-- The mouse homolog of clone 35 was also isolated and sequenced (FIG. 5 and see SEQ ID NO: 15). The mouse nucleotide sequence differs in 35 positions relative to the rat sequence and contains 16 additional nucleotides near its 3' end. Of these differences, 19 nucleotides differ within the protein coding region. Only 7 of these affect the encoded protein sequence. One amino acid difference is a neutral substitution in the secretion signal sequence (residue 3). The remaining 6 differences are in the C-terminal region. One of these obliterates a potential proteolytic cleavage site. This observation and the nature of the other differences make it unlikely that 2 of the possible maturation products of the rat preproprotein are functional. However, the 2 peptides that are related both to each other and to secretin are absolutely preserved between species, providing strong support for the notion that these peptides have a function conserved during evolution. --

Rewrite the paragraph beginning on page 52, line 19, to read:

-- Antibodies directed to a conserved epitope common to the hypocretin polypeptides of several species will detect hypocretin polypeptides of mammalian species in general. For example, antibodies directed against such a conserved sequence as GNHAAGILT (SEQ ID NO: 13; Fig. 5B) can be used to detect human hypocretin polypeptides. --

Rewrite the paragraph beginning on page 71, line 4, to read:

-- Subtractive hybridization was performed in two cycles using the previously described procedure (Usui et al., *supra*). Briefly, 1 µg of trace-labeled, tagged

hypothalamus target cDNA prepared as described from the pT7T3D target library was annealed for 24 hrs at 68 degree Celsius in 10 μ l of hybridization buffer (Usui et al, *supra*) with 20 μ g cerebellum cRNA (ratio 1:20). After hydroxyapatite chromatography, the single-stranded fraction corresponded to 10% of the input material, as judged by tracer quantitation. This was mixed with 20 μ g of hippocampus cRNA (estimated ratio 1:200) for a second 24 hr hybridization, after which 30% of the input chromatographed at the single-strand position. Cumulatively, these steps removed more than 97% of the input tracer. An aliquot of the single-stranded material was used as template in a 30-cycle PCR (program: 94 degree Celsius for 15 sec, 60 degree Celsius for 15 sec, 72 degree Celsius for 1 min) using primers corresponding to the tag sequences (Usui et al., *supra*):

5'-AACTGGAAGAATTCGCGG-3' (SEQ ID NO: 19) and
5'-AGGCCAAGAATTCGGCACGA-3' (SEQ ID NO: 16). The amplification product was cleaved with NotI, then EcoRI, and inserted into pBCSK⁺. A dot blot was prepared and screened with probes prepared from the target, subtracted target and driver libraries as previously described by Usui et al, *supra*, using serial dilutions of plasmid cDNA clones isolated previously in this laboratory. The target and subtracted target cDNA libraries were screened to determine the frequency of oxytocin and VAT-1 cDNA clones using as probes clones isolated in the present study. --

Rewrite the paragraph beginning on page 72, line 2, to read:

-- Similarly, following the procedures of Example 1, a mouse (C57/B16) hypothalamus cDNA library, constructed in the pT7T3D vector, was used as a template for PCR amplification (primers 5' TAAGACGACGGCCTCAG 3', SEQ ID NO: 17, and 5' CACACCAACAGAGAAACG 3', SEQ ID NO: 18) to obtain the mouse homolog of the rat H35 cDNA obtained above. The mouse and rat cDNA (SEQ ID NO: 15 and SEQ ID NO: 14, respectively) and protein sequences (mouse SEQ ID NO: 2; rat SEQ ID NO: 1) are compared in Fig. 5. The 569 nucleotide rat sequence has the potential to encode a 130-residue putative secretory protein (preprohypocretin) with an apparent signal sequence and 3 additional sites for potential proteolytic maturation (Fig. 5A). Two of the putative

products of proteolysis (hert1, SEQ ID NO: 7 and hert2, SEQ ID NO: 9) have 14 amino acid identities across 20 residues (Figs. 5B). This region of one of the peptides contains a 7/7 match with secretin (Fig. 5B, SEQ ID NO: 21), suggesting that the prepropeptide gives rise to two peptide products that are structurally related both to each other and to secretin. --

Rewrite the paragraph beginning on page 72, line 15, to read:

-- The mouse hypocretin nucleotide sequence (SEQ ID NO: 15) differs in 35 positions relative to the rat (SEQ ID NO: 14), and contains 16 additional nucleotides near its 3' end. Of these differences, 19 are within the putative protein-coding region (Fig. 5A), only 7 of which affect the encoded protein sequence: one amino acid difference at residue 3 is a neutral substitution in the apparent secretion signal sequence; the remaining 6 differences are near the C-terminus, one of which obliterates a potential proteolytic cleavage site. The absence of this site and the nature of the other differences make it unlikely that two of the four possible rat maturation products are generated and functional in mice. However, the two putative hert peptides that are related both to each other and to the secretin family are absolutely preserved between the two species, providing strong support for the notion that these peptides have a function conserved during evolution. Both hert1 and hert2 terminate with glycine residues, leaving the nitrogen of the terminal glycine as a C-terminal amide in the mature peptide. --

Rewrite the paragraph beginning on page 73, line 26, to read:

-- The respective 5' and 3' primers, 5' ATCGAGATCTAGACACCATG AACCTTCCTTCTACAAAGGTT 3' (SEQ ID NO: 22) and 5' ACTGTCTA GATCATAGATCTTCTTCAGAAATAAGTTTTGTTCTGACTCTGGATCCGCCCCGGG GCGCT 3' (SEQ ID NO: 23), are used as primers to amplify H35 beginning at position 85 in SEQ ID NO:3 with an inserted BglIII site added at its 5' end to the 3' end having an inserted c-myc epitope tag. The PCR products are subcloned into pCMV and transfected into a mammalian host cell to produce an H35-myc tagged protein product. --

Rewrite the paragraph beginning on page 74, line 4, to read:

12.2
-- H35 proteins are also produced in bacteria by subcloning the H35 coding sequence into pRSET B (Invitrogen, San Diego, CA), which encodes six histidines prior to the H35 sequence. The vector contains a T7 promoter which drives expression of 6XHis-tagged proteins in *E. coli*. The respective 5' and 3' oligonucleotides
5' ATCGAGATCTCTTGGGGTGGACGCGCAGCCT 3' (SEQ ID NO: 24) and
5' ACTGAATTCTCAGACTCTGGATCCGCCCCG 3' (SEQ ID NO: 25) are used as PCR primers to amplify the rat H35 sequence into the BglII and EcoRI sites of the pRSET B vector. The resulting hypocretin-poly-(His) fusion protein may be purified by affinity chromatography on a metal affinity resin. --

Rewrite the paragraph beginning on page 74, line 16, to read:

13.13
-- The mouse *Hcrt* gene was mapped to Chromosome 11 using an interspecific backcross. A single-strand sequence polymorphism between C57BL/6J and SPRET/Ei was detected as previously described and mapped on The Jackson Laboratory BSS panel. An *Hcrt*-specific product of approximately 600 base pairs was amplified from mouse C57BL/6J genomic DNA using synthetic oligonucleotides
5'-GACGGCCTCAGACTTCTTGG-3' (SEQ ID NO: 26) and
5'-GCAACAGTTCGTAGAGACGG-3' (SEQ ID NO: 27). This product contained a putative intron, and its identity as *hcrt* was confirmed by sequencing (data not shown). Genotype data and references for these and other linked markers can be accessed via the Mouse Genome Database (<http://www.informatics.jax.org>). --

Rewrite the paragraph beginning on page 75, line 15, to read:

14.1
-- A polyclonal antiserum (serum 2050) was raised to a chemically synthesized peptide corresponding to the C-terminal 17 amino acid residues (CPTATATACAPRGGSRV, SEQ ID NO: 28) of the rat preprohypocretin sequence. In Western transfer blots using as target electrophoretically separated proteins from bacteria transformed with the plasmid pRSET B engineered to express preprohypocretin, a single prominent immunoreactive band was observed with a migration of approximately 19kDa

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with the hyperimmune serum, but not with the preimmune serum. No immunoreaction was detected with an extract from bacteria transformed with a preprohypocretin/pRSET B expression plasmid, indicating that detection of the 19kDa target requires hypocretin expression. Analogous results were obtained with an additional antiserum to the 17mer and two antisera to synthetic hcrt2. --

Please also amend the specification to enter the attached paper copy of the substitute Sequence Listing.

In the Drawings:

Amend FIGURE 5A as described herein and in the appended marked-up figure.